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Cyanogenic *Pseudomonas* spp. strains are concentrated in the rhizosphere of alpine pioneer plants

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ABSTRACT

HCN producing bacteria have previously been isolated from alpine mineral soil and their ecophysiology was presumed to be associated with mineral weathering. Nevertheless, the high ecological patchiness of the alpine environment calls for an extensive and detailed analysis of the spatial distribution of HCN producing bacterial populations and their associated weathering traits. Our results of such an analysis showed that primarily the rhizosphere of pioneer plants was rich in HPPs, harbouring the most potent HCN producers. HCN production incidence and intensity were dependent on the plant-associated micro-habitat and type of bedrock/mineral soil, however the HCN+ phenotype was not associated with one of the particular genotypes which we determined by BOX-PCR. In HPP isolates, HCN production most commonly co-occurred with the production of hydroxamate-type siderophores, but was less often associated with inorganic phosphate solubilization activity and the production of catechol-type siderophores. These observations indicate that a plant's physiotype, not species, provide physicochemical conditions that determine selective pressure, which enables the growth of *Pseudomonas* spp. with a random genotype, but phenotypically predetermined to increase mineral weathering via a particular combination of phosphate solubilization and iron complexation with siderophores and HCN.

1. Introduction

Biogenic cyanogenesis has been found in organisms of all kingdoms except Archaea. Generally, cyanide (HCN) production is carried out by two mechanisms: (*i*) in plants (Poulton, 1990; Vetter, 2000) and insects (Nahrstedt, 1988) via the degradation of cyanogenic glycosides and (*ii*) in bacteria (Blumer and Haas, 2000) and fungi (Stevens and Strobel, 1968) by the *de novo* synthesis of HCN. Since it has been widely accepted that the ecological role of cyanogenic glycosides in plants serves for inter- and intra-species competition through the inhibition of grazing organisms, a similar reasoning has been adopted to explain the role of HCN production in microorganisms. Since HCN producing bacteria (HPB) have been isolated from different environments, such as the lungs of cystic fibrosis patients (Enderby et al., 2009; Goldfarb and Margraf, 1967) and the plant rhizosphere, HCN production was on the one hand recognized as a pathogenic factor acting against the host (Winstanley and Fothergill, 2009) and on the other as the host's biocontrol or protecting agent (Haas and Défago, 2005).

Although HCN production was found in several bacterial species, available data predominately connected it to HPPs. Current molecular data on HCN synthase deposited at the GenBank® Gene and Nucleotide databases associate HCN synthesis primarily with pseudomonads, since past studies focused mostly on this group of bacteria, and because they actually are the predominant group that produces HCN in the environment. Soil fluorescent pseudomonads have been extensively studied (reviewed in Blumer and Haas, 2000), focusing on *hcnABC* operon genetics (Laville et al., 1998), pseudomonad ecological association with plants (Ramette et al., 2003) and the biocontrol of plant pathogens by HPPs (reviewed in Haas and Défago, 2005). The presumed role of HCN as a biocontrol agent was most widely studied. HCN was shown to inhibit (*i*) several fungal root diseases, (*ii*) the development of disease symptoms in seedling leaves and (*iii*) the growth of several phytopathogenic fungi *in vitro* (Blumer and Haas, 2000). However, no evidence is currently available demonstrating that the inhibition

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Abbreviations: HCN+/−, HCN positive/negative phenotype; HPB, HCN producing bacteria; HPP, HCN producing pseudomonads; LOA, low-molecular weight organic acid; PGP, plant growth promoting; KB, King's B; LB, Luria-Bertani.

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of phytopathogens in soil and rhizosphere happens due to bacterial HCN production *in situ*. Furthermore, it has been demonstrated in some cases that bacterially produced HCN could cause phytotoxicity (Blom et al., 2011; Rudrappa et al., 2008) which raised the question, whether biocontrol is in fact the ecological role of bacterial HCN production.

One possible alternative hypothesis for HCN action is in the regulation of micronutrient availability. HCN produces stable complexes with transient metals, like Fe, Zn, Cu, etc. (Rennert and Mansfeldt, 2002; Wongfun et al., 2014) which has recently become a topical issue in oligotrophic environments, such as alpine regions and glacier forefields (Lapanje et al., 2012). The extent of alpine oligotrophic environments is steadily increasing and bare bedrock surfaces are becoming more exposed due to continuous water erosion (Hales and Roering, 2005) and the retreat of glaciers (Cook et al., 2005; Kaser et al., 2004). The newly exposed bedrock gets only slowly populated by different living organisms due to low nutrient availability (Lazzaro et al., 2009). In this respect, the key organisms that speed up raw bedrock dissolution and help form new soil material, are the pioneering plant species and their rhizospheric microbial communities. Plants alone already produce low-molecular weight organic acids (LOAs), like oxalic and citric acid, to solubilize minerals (Ström et al., 1994), but an additional impact is caused by plant-associated bacteria and their plant growth promoting (PGP) traits, like the production of siderophores (Selvakumar et al., 2008), the solubilization of phosphate (Pandey et al., 2006) and the production of HCN (reviewed in Hayat et al., 2010).

Since these traits acting on the same target (e.g. Fe) (Frey et al., 2010; Rennert and Mansfeldt, 2002; Wongfun et al., 2014) are efficient in different conditions (e.g. pH) and because plants also contribute to the release of nutrients as well as compete for their uptake, we expected to see these traits would be differentially distributed in HPPs from oligotrophic alpine environments like granite (low pH) or limestone (high pH) mineral soils. Since soil pH can affect the interaction between transition metals and HCN due to the high pK value of HCN, this should reflect on the ecology of HPP and their potential for HCN production. Based on the results of our alpine sampling campaigns, showing that HPB were common in the rhizosphere of pioneer plants, particularly those with known weathering activity (Supplementary data – Table S1), we focused our experiments on this plant physiotype.

According to current knowledge, here for the first time we have addressed, the idea that the ecophysiological weathering characteristics of HPPs are reflected on the population level of pseudomonads isolated from different alpine plant-associated microhabitats. Since we had to screen a large library of *Pseudomonas* spp. isolates, we used basic, well-established, quantitative and semiquantitative microbiological and biochemical techniques as well as molecular genotyping. The specific aims of the study were to: (*i*) determine the pattern of distribution of HCN producing (HP) isolates in different microhabitats, soil types and host plant species, (*ii*) investigate, if HCN production is associated with isolate origin or strain genotype and (*iii*) determine, according to our hypothesis, the correlation between HCN production and other mineral weathering traits, namely the production of different types of siderophores and the capability to solubilize phosphorates.

2. Materials and methods

2.1. Environmental sampling

Environmental samples were collected from silicate (granite) based mineral soil and gravel material on the Damma glacier forefront (altitude 2100 m a.s.l., 46°38.177′ N, 008°27.677′ E), Switzerland, and the calcareous (limestone) mineral soil and gravel material on the mountain scree by Lake Krn (altitude 1400 m a.s.l., N46.282598, E13.681625), Slovenia (Supplementary data – Fig. S1). We collected samples of plant-unassociated mineral soil as well as samples of the rhizosphere material and plant roots of alpine pioneering plants *Oxyria dygina* and *Leucanthemopsis alpina* (both from the granite-based location) and *Rumex scutatus* and *Dryas octopetala* (both from the limestone-based location). Altogether we obtained 20 rhizosphere and root samples from granite-based environment (10 samples each), 26 rhizosphere and root samples from limestone-based environment (13 samples each) and 3 samples of plant-unassociated mineral soil from limestone-based environment. All samples were transported to the laboratory in chilled transport containers and were stored at 4 °C for up to 3 days prior to strain isolation.

2.2. Isolation of bacterial strains

On site, the collected rhizosphere material was additionally cleaned using sterile tweezers of any root part residue and vice versa for root samples. Each amount of soil, rhizosphere and root material was weighed (weight range 1–5 g) and was washed thoroughly by vortexing in 3 ml of sterile 0.9% NaCl. The obtained solution was 10-fold diluted up to 10^{-9} and 100 μl was spread on King's B agar plates (20 g L^{-1} peptone, 1.5 g L^{-1} K_2HPO_4, $MgSO_4 \cdot 7H_2O$, 10 ml L^{-1} glycerol, 15 g L^{-1} agar). The plates were incubated at 25 °C for 14 days to determine the number of culturable bacteria by colony counting. From these primary plates up to ten pseudomonas-like colony phenotypes (slimy or production of diffusible fluorescent pigment) were isolated. Pure cultures were stored at −80 °C prior to further analysis. To confirm the identity of the pseudomonads, DNA of sixteen random isolates was obtained using Chelex® 100 Resin (Walsh et al., 1991), 16S rRNA gene was PCR amplified using standard 27f and 1495r primers (Supplementary data – Table S2) according to (Hubad and Lapanje, 2013) and was sent for sequencing (Macrogen, Netherlands). The identity of all sixteen strains was determined to be *Pseudomonas* sp. using Seqmatch (Version 3) of the RDP project (Release 11_4, Maidak et al., 2001) (Supplementary data – Text S1).

2.3. Characterization of isolates

The bacterial strains were characterized according to (*i*) HCN production, (*ii*) weathering potential, namely, $Ca_3(PO_4)_2$ solubilization activity, production of fluorescent pigment pyoverdine and the production of a variety of siderophores other than pyoverdine, and (*iii*) by BOX-PCR based genetic fingerprinting.

2.3.1. HCN production

HCN production was determined per cell number. The cell numbers in the liquid media were determined by the conversion of the five-parametric logistic curve fit of the OD_{600} values corresponding to particular cell numbers of *Pseudomonas fluorescens* strain CHA0 (Laville et al., 1998) determined by colony counting. Free CN^- ions in inoculated media supplemented with glycine (5 g L^{-1}) were measured by using the methemoglobin-based colorimetric method (von Rohr et al., 2009). The concentration calibration curve was based on a series of freshly prepared KCN (Alfa Aesar, Germany) concentrations from 25 μM to 0 μM with 5 μM increments. The measurements were performed in a clear bottom 96 well Microplates (Greiner Bio-One, Germany) using the SynergyH4 multi-plate reader (Biotek, USA).

2.3.2. Phosphate solubilization

Freshly grown colonies on nutrient agar (Sigma-Aldrich, USA) of each of the bacterial strains were transferred to Pikowskaya